Soil Microbial Communities Associated with Bt and Non-Bt Corn in Three Soils

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ABSTRACT

The effects of expression of Cry endotoxin by Bt corn (transgenic corn engineered to express *Bacillus thuringiensis* toxin) on soil microbial community structure were assessed in a growth chamber experiment. Two lines of transgenic corn expressing different Cry endotoxins were compared with their respective non-transgenic isolines in three soil types with differing textures. Phospholipid fatty acid (PLFA) profiles from bulk soil and community-level physiological profiles (CLPP) from the rhizosphere community were used to assess community structure. Differences in PLFA profiles due to soil type were significant, accounting for 73% of the total variability in the dataset. Differences in bacterial and fungal CLPP profiles due to soil type were statistically significant, but probably not biologically important, accounting for 6.3 and 3.8% of the total variability, respectively. Neither expression of Cry endotoxin nor corn line had a significant effect on microbial profiles, except in the high-clay soil where both factors significantly affected bacterial CLPP profiles (accounting for 6.6 and 6.1% of the variability in that soil, respectively). Expression of Cry endotoxin also significantly reduced the presence of eukaryotic PLFA biomarker in bulk soils, although it is unclear which groups of eukaryotes were affected. We conclude that the effects of transgenic Bt corn in this short-term experiment are small, and longer-term investigations are necessary.

Soil microorganisms will come into direct contact with transgenic Cry endotoxin as it is released from Bt corn and other crops in root exudates and from decomposing tissue (Palm et al., 1996; Saxena et al., 2002a). While *Bacillus thuringiensis* occurs naturally in soil, growth of transgenic Bt corn causes a large increase in the amount of Cry endotoxin present in agricultural systems [roughly 0.25 g/ha produced naturally (calculated from approximately 1000 *Bacillus thuringiensis* spores/g soil; Phyllis Martin, personal communication) versus 650 g/ha in a Bt corn crop, excluding grain (calculated from USEPA, 2000, 2001)]. The soil microbial community is an integral component of soil quality, so it is essential that the impact of this new input into agricultural systems on the community is assessed. Another mechanism by which Bt corn could alter soil microbial communities is through increased lignin concentration (Saxena and Stotzky, 2001b). Lignin content is a primary predictor of plant residue decomposition rate (Parton et al., 1996), and decomposition of lignin itself can be performed by only a small set of soil organisms.

The effects on soil microbial community composition of expression of Cry endotoxin specifically by corn have not been investigated, although several studies have examined the effects of transgenic Cry endotoxins on numbers of culturable microorganisms. Saxena and Stotzky (2001a) found no changes in numbers of culturable bacteria or fungi due to presence of Cry1Ab endotoxin in corn root exudates and decomposing shoot tissue. Increased populations of culturable bacteria and fungi were found in microcosms with decomposing cotton (*Gossypium hirsutum* L.) tissue containing Cry1Ac endotoxin (Donegan et al., 1995) and on leaves of transgenic potato (*Solanum tuberosum* L.) producing Cry3 endotoxin (Donegan et al., 1996). These results indicate that the Cry endotoxins will not reduce overall numbers of culturable microorganisms, but it should be recognized that the vast majority of soil microorganisms are not culturable (Torsvik et al., 1990); in fact several phyla of eubacteria known from molecular studies to be common in soil have rarely, if ever, been cultured from the habitat (Hugenholtz et al., 1998). Also, changes in the relative abundances of species (i.e., changes in community composition or structure) are missed using plate counts of culturable organisms, implying that reliance on cultivation to detect impacts of anthropogenic chemicals on microbial communities is inadequate (Johnsen et al., 2001). In the only study to have looked for responses in soil microbial community composition to Cry endotoxins, Donegan et al. (1995) found transient differences between communities from microcosms containing decomposing Bt and non-Bt cotton. Community structure was assayed by amplified ribosomal DNA restriction analysis (ARDRA) and community-level physiological profiling (CLPP), and differences were assessed by inspection of gels and principal components plots of CLPP data, rather than by explicit hypothesis-testing methods.

In this study we investigated the effects of transgenic corn expressing European corn borer active Cry endotoxins, compared with corresponding non-transgenic isolines, on the community structure of soil microbes. The experiment was performed using two corn lines transformed with different Cry endotoxins. Since soil type has been shown to affect the dynamics of Cry endotoxin in soil (Tapp and Stotzky, 1998), the experiment was conducted in three soils with differing textures. Community structure was characterized by phospholipid fatty acid (PLFA) profiles. This method has previously been used to detect changes in microbial communities due to agronomic management (e.g., Zelles et al., 1995; Bossio et al., 1998). In addition, CLPP was used to assay the catabolic potential of bacterial and fungal communities with respect to 95 carbon substrates (Garland and Mills, 1991; Buyer et al., 2001).

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Abbreviations: Bt corn, transgenic corn engineered to express *Bacillus thuringiensis* toxin; CLPP, community-level physiological profile; PLFA, phospholipid fatty acid.

MATERIALS AND METHODS

Soils

Soil was collected from University of Maryland Agricultural Experiment Station sites in Clarksville, Salisbury, and Upper Marlboro, MD, in October 2001. All fields had been planted to non-Bt corn. Soils were mixed and plant residue removed by sieving over a 9.5-mm sieve. Soil texture was determined by soil hydrometer after dispersal of soil with sodium hexametaphosphate and blending. Soil pH was measured after saturation of a sample with $CaCl_2$. Total C and N were measured by combustion in a Carlo Erba (Milan, Italy) C/N analyzer.

Growth Chamber Experiment

The growth chamber experiment was designed as a three (soils) × two (corn lines) × two (Cry protein expression) complete factorial with eight replicate pots per treatment. One pot for each treatment was randomly assigned a position in each of eight racks, which served as experimental blocks. Round plastic pots (25.4-cm-deep × 7-cm-wide Conetainers; Stuewe and Sons, Corvallis, OR) were filled with approximately 500 mL of soil after plugging with cotton fiber. Two sweet corn seeds were planted approximately one inch deep. Seeds used included Hybrid Prime Plus F1 with or without the Bt11 event causing expression of Cry1A(b) (Syngenta Seeds, Boise, ID), and a line with or without the TC1507 event causing expression of Cry1F (Pioneer Hi-Bred International, Des Moines, IA). Seeds were provided by Dr. Galen Dively from the University of Maryland.

Plants were grown at 20°C under 12 high-intensity fluorescent lights and eight 100-W incandescent lights. Watering was every other day with distilled water until seeds germinated and were thinned to one seedling per pot (less than 7 d). Plants then received 0.5X Hoaglands solution [2.5 m*M* $Ca(NO_3)_2$/KNO_3, 1 m*M* K_2HPO_4/$MgSO_4$, 25 μ*M* H_3BO_3, 10 μ*M* $FeSO_4$/Na_2EDTA, 5 μ*M* $MnCl_2$, 2 μ*M* $ZnSO_4$, 1 μ*M* $CuCl_2$, 0.2 μ*M* $CoCl_2$/$NiSO_4$/$(NH)_6Mo_7O_{24}$] every other day until 24 d after planting. At this point plants appeared slightly stressed so watering was increased to every day with 2.5X Hoaglands solution. At 27 d after planting, corn plants no longer appeared stressed, but urea was used to replace $CaNO_3$ and K_2HPO_4 due to poor soil drainage from $CaPO_4$ precipitated from the nutrient solution.

Sampling

Plants were harvested 36 d after planting. Roots and adhering soil approximately 2 mm or less in diameter were separated from bulk soil by gently shaking the root system and breaking larger aggregates along planes of weakness. We will use the term "rhizosphere" to describe these samples, which includes the rhizoplane and roots. Subsamples of bulk soil and rhizosphere were taken for determination of moisture content, and of rhizosphere for CLPP, and the remainder was stored at −20°C. Shoot fresh weight was determined. Shoots were air-dried and re-weighed periodically until their weight stabilized (8 wk). One replicate (Clarksville soil, Syngenta line, Cry expressed) was not analyzed due to severely stunted plant growth.

Phospholipid Fatty Acid Profiles

Lipids were extracted from bulk soil using a modified Bligh–Dyer procedure (Kehrmeyer et al., 1996). Five grams of lyophilized soil were placed in a 25-mL centrifuge tube to which was added 4 mL phosphate buffer, 5 mL chloroform, and 10 mL methanol. The mixture was sonicated for 10 min and shaken for 2 h. Following centrifugation at 1800 × *g* for 10 min, the liquid phase was transferred to a 30-mL test tube. Chloroform and water (5 mL each) were added and the phases were allowed to separate overnight. The aqueous phase was removed and the organic phase evaporated under nitrogen.

The lipids were fractionated by column chromatography using a 500-mg silica gel SPE column. The column was preconditioned with chloroform and then loaded with the sample in chloroform. Lipids were sequentially eluted with chloroform (5 mL), acetone (10 mL), and methanol (5 mL). The methanol fraction, containing the phospholipids, was evaporated under nitrogen.

Transesterification of the phospholipids was accomplished as previously described (Petersen and Klug, 1994). The resulting fatty acid methyl esters were dissolved in 200 μL of 1:1 hexane to methyl t-butyl ether and analyzed by gas chromatography. An Agilent 6890 gas chromatograph (Agilent Technologies, Palo Alto, CA) was used to identify and quantitate fatty acid methyl esters according to the MIDI eukaryotic method (Microbial ID, Inc., Newark, DE). The PLFA identities were confirmed in several randomly chosen samples by gas chromatography–mass spectrometry using an Agilent 5890 gas chromatograph and an Agilent 5970 mass spectrometer.

Community Level Physiological Profiles

Approximately 4 g rhizosphere per sample was diluted with a volume of sterile water equal to nine times its fresh weight (10^{-1} dilution) and sonicated for 5 min, and an aliquot of this was further diluted to 10^{-2}. Fungal physiological profiling was performed as described by Buyer et al. (2001). A 0.15-mL aliquot of the 10^{-1} dilution was mixed with 14.85 mL of 0.2% agar containing 100 μg/mL of both rifampicin and streptomycin and 50 μg/mL gentamycin. One-hundred microliters of this agar solution was then inoculated into each well of a Biolog (Hayward, CA) SF-N plate. For bacterial physiological profiling, a 0.25-mL aliquot of the 10^{-2} dilution was mixed with 24.75 mL sterile saline. One-hundred-fifty microliters was then inoculated into each well of a Biolog GN plate. Plates were incubated at 22°C. Absorbance was measured once per day on a microplate reader at 595 nm beginning on Day 3 of incubation for bacterial plates, and at 650 nm beginning Day 5 for fungal plates. Plates were incubated until the average absorbance across wells of a plate reached 1.0 for bacteria and 0.5 for fungi, or until Day 12 of incubation.

Statistical Analysis

The effects of experimental factors on community profiles were tested by redundancy analysis in the program Canoco (Microcomputer Power, 1998). This procedure is a canonical version of principal components analysis and generates *P* values based on the proportion of the total variance explained by each experimental factor. The empirical proportions are compared with a distribution of values created by 9999 random permutations of the profiles. Indicator variable(s) of the factor being tested served as the environmental variables, while indicator variables for other factors were covariables (Legendre and Anderson, 1999). Shoot dry weight was also tested to determine whether there was a significant correlation with community composition, and whether it had any effect as a covariable in testing experimental factors. Values for the one lost replicate were replaced by the mean of the other replicates of that treatment as per suggestions in Legendre and Anderson (1999). Datasets were checked for outliers using raw data canonical principal components plots (i.e., not group means).

Redundancy analysis of PLFA profiles was performed on

chromatographic areas of fatty acid methyl esters, mol % (relative proportions of peak areas), and after the Hellinger transformation (square root of relative proportions) recommended by Legendre and Gallagher (2001) for analysis of ecological communities. In separate analyses, total PLFA extracted and biomarkers for various groups of organisms were analyzed by analysis of variance using Proc Mixed in SAS (SAS Institute, 2001), with experimental block designated as a random effect. The fatty acid 16:0 was used as an external standard to convert chromatographic area to nmol PLFA/g dry soil. Biomarkers included polyunsaturated PLFAs for eukaryotes, iso and anteiso branched PLFAs for Gram positive bacteria, 18:2 ω6c for fungi, 20:3 ω6c and 20:4 ω6c for protozoa, 16:1 ω5c for arbuscular mycorrhizal fungi, and 15:0, iso 15:1, iso and anteiso 17:1, iso 17:1 ω5c, cyclo 17:0, and cyclo 19:0 for eubacteria (Buyer et al., 2002).

Analysis of CLPP was conducted on the integrated area under the curve of color development for each well over a defined time period (3 to 8 and 5 to 8 d for bacterial and fungal plates, respectively; calculated as in Hackett and Griffiths, 1997). This was compared with analysis of single plate readings where average well-color development was closest to a target value (1.0 and 0.5 for bacterial and fungal plates, respectively). Results of the latter method were similar and are not shown. The value for the blank well of each plate was used as a covariable in redundancy analysis.

RESULTS

Soil chemical and physical properties are shown in Table 1. Clarksville soil yielded an average of 77.2 nmol PLFA/g dry soil, UM soil yielded 41.5, and Salisbury soil yielded 25.7. Analysis of total PLFA yields was performed after log transformation to correct for unequal treatment variances. Differences in total PLFA extracted between soils were significant ($p < 0.0001$), but effects of Cry proteins, corn line, and interactions were not ($p > 0.1$).

Log abundance of PLFA biomarkers was primarily affected by soil ($p < 0.0001$), following the trends described for total PLFA. Other significant treatments included a decrease in eukaryotic biomarkers in pots with plants expressing a Cry protein (2.8 nmol/g) compared with non-Cry plants (3.3 nmol/g; $p = 0.045$). An increase in levels of the eubacterial and Gram positive biomarkers was associated with Pioneer corn in Clarksville soil compared with Syngenta corn in Clarksville soil (test of line–soil interaction; $p = 0.016$ for eubacteria and $p = 0.040$ for Gram positives).

Soil type significantly affected both CLPP and PLFA profiles (which includes all PLFAs detected, not just biomarkers) (Table 2). While significant, the magnitude of the soil effect was quite small on CLPP. In canonical principal components plots derived from the redundancy analyses, much greater separation between soil types is evident for PLFA than for CLPP (Fig. 1 and 2, respectively). The soil effect was larger for analysis of absolute PLFA peak areas compared with Hellinger-transformed peak areas (Table 2) because absolute peak area is sensitive to differences in the total amount of PLFA extracted from the soil. There was no overall effect of expression of Cry proteins or corn line on PLFA or CLPP (Table 2). One bacterial CLPP outlier was identified but deletion of this profile did not affect the analysis. Plant shoot weight was not a significant covariable ($p > 0.1$). Results of redundancy analysis on mol % PLFA data were similar to those described above, with 43% of the total variability explained by soil type (not shown).

Interaction effects were only significant in the bacterial CLPP (Table 2). The cause of this significance was determined by examination of raw data canonical principal components plots (treatment means are shown in Fig. 1 and 2 because of the large number of datapoints). The major interaction effect evident was a separation between Bt and non-Bt corn in the Clarksville communi-

Table 1. Soil properties.

Soil	pH	C	N	Clay	Silt	Texture
		%				
Clarksville	5.9	1.59	0.14	42	34	clay
UM	5.3	0.66	0.06	25	18	sandy clay loam
Salisbury	5.7	0.32	BDL†	10	8	loamy sand

† Below detection limit.

Table 2. Redundancy analysis of community-level physiological profiles (CLPP) area and phospholipid fatty acid (PLFA) profiles.

Treatment	CLPP		PLFA	
	Bacteria	Fungi	Absolute†	Hellinger‡
	%§			
Bt	0.6	1.0	0.5	0.9
Soil	6.3***	3.8**	73***	29***
Line	0.9	1.0	0.2	0.7
Interaction	7.7*	6.4	2.9	5.8

* Significant at the 0.05 probability level.
** Significant at the 0.01 probability level.
*** Significant at the 0.001 probability level.
† Phospholipid fatty acid peak areas.
‡ Hellinger-transformed PLFA peak areas as described in text.
§ Proportion of total variance explained by the experimental factor.

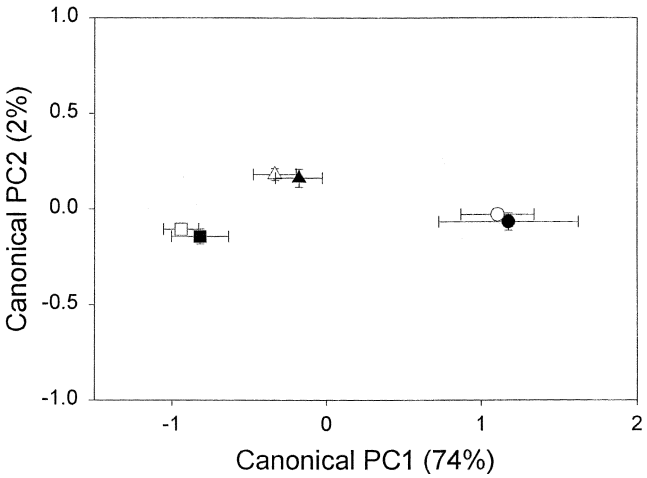

Fig. 1. Canonical ordination of phospholipid fatty acid (PLFA) profiles derived from redundancy analysis of absolute peak areas. Percentages on axes are percent of total variation in dataset explained; 99% of the variability that can be attributed to experimental factors is shown by this plot. Symbols represent treatment means and bars are 95% confidence intervals for each treatment (corn line treatments have been pooled). Triangles, UM soil; squares, Salisbury soil; circles, Clarksville soil; white, Bt corn; black, non-Bt corn. PC, principal component.

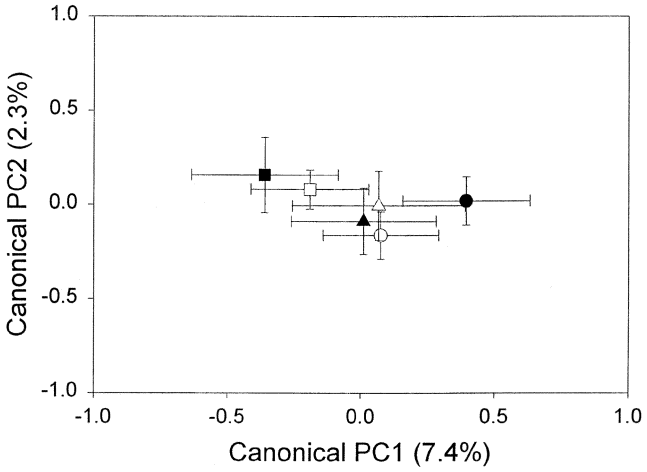

Fig. 2. Canonical ordination of bacterial community-level physiological profiles (CLPP) profiles derived from redundancy analysis. Plot details are as described for Fig. 1; 62% of the variability that can be attributed to experimental factors is shown by this plot. PC, principal component.

ties. To analyze this interaction further, redundancy analysis of bacterial CLPP was re-run on Clarksville soil only. Both Cry protein and corn line were found to be significant ($p < 0.01$), accounting for 6.6 and 6.1% of the total variability in Clarksville bacterial CLPP, respectively (Fig. 3). There was no significant interaction effect. Color development in several wells containing amino acids or other amine-containing substrates were positively associated with Cry protein (see Fig. 3 for substrates). Substrates that differentiated between Syngenta and Pioneer corn lines included mostly carbohydrates and carboxylic acids.

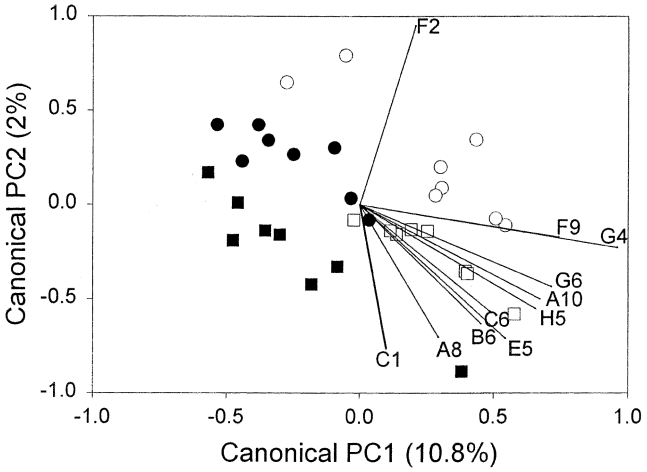

Fig. 3. Canonical ordination of Clarksville soil community-level physiological profiles (CLPP) profiles derived from redundancy analysis. Percentages on axes are percent of total variation in dataset explained; 81% of the variability that can be attributed to experimental treatments is shown by this plot. Lines represent substrate vectors showing their contributions to the ordination. Only substrates with >22% of variance explained by the experimental treatments are shown. Each vector label corresponds to the well containing the substrate in GN Biolog plates; A8 = *N*-acetyl-D-glucosamine, A10 = L-arabinose, B6 = α-D-glucose, C1 = D-melibiose, C6 = D-sorbitol, E5 = α-keto valeric acid, F2 = succinamic acid, F9 = L-aspartic acid, G4 = L-ornithine, G6 = L-proline, H5 = phenyl ethylamine. Symbols represent positions of individual replicate profiles. Squares, Pioneer corn; circles, Syngenta corn; white, Bt corn; black, non-Bt corn. PC, principal component.

DISCUSSION

The large effect of soil type found in this experiment was not unexpected, and has previously been shown to be the major determinant of soil microbial community structure in agricultural systems (Bossio et al., 1998; Girvan et al., 2003) or growth experiments (Buyer et al., 1999, 2002). Soil type probably affects both the initial community composition and the conditions for growth during an experiment.

There were very few significant effects of the expression of lepidopteran-active Cry proteins on soil microbial communities in this growth chamber experiment. In the Clarksville soil the effect of Cry protein on bacterial CLPP was statistically significant, but the amount of variability accounted for was small. In this soil, presence of Cry protein seemed to stimulate increased ability of the rhizosphere community to metabolize certain amino acids and other amines. The only reports on protein content and amino acid composition of Bt and non-Bt corn that we are aware of claim that there are no differences in corn expressing Cry1F and limited differences in corn expressing Cry9C (USEPA, 2000, 2001). It has been shown that Cry1Ab protein is released from corn roots during growth (Saxena et al., 2002a). It is unknown whether Cry proteins replace other root exudate proteins, or are exuded in addition to normal root exudates, thereby increasing the total amount of protein exuded. The increased use of certain amino acids and amines in Clarksville soil with no concomitant decrease in the use of other compounds (Fig. 3) suggests that Bt corn simply caused proliferation in the population of certain microorganisms due to extra protein in the environment. The presence of a small Bt effect in the Clarksville soil and not in the other soils is probably due to the high clay content of Clarksville soil. Clay has been shown to increase retention of Cry protein in soil (Saxena et al., 2002b), allowing for an extended period of exposure to microbes in the rhizosphere of higher clay soil.

In contrast to CLPP, there were no significant effects of expression of Cry protein on PLFA profiles. The overall effects of Cry protein in CLPP were small, and it is possible that small in situ changes to the community were amplified during the in vitro incubation of Biolog plates, such that changes in community structure were not detected using PLFA analysis, which is a more broad-scale, instantaneous measure of community structure. However, the differences in conclusions reached between these two methods could also be due to the fact that they were used in differing soil microhabitats, as appropriate for the communities inhabiting them and the methods' inherent limitations. The CLPP characterizes those members of a community that are fast-growing aerobic heterotrophs, which are stimulated in, and an important component of, the rhizosphere (Buyer et al., 2002). The PLFA profiles provide an analysis of all organisms present, and was therefore not used in the rhizosphere in this experiment because it was possible

that different plant phospholipids could confound the experimental treatments. There was a significant reduction in the eukaryotic PLFA biomarker in bulk soils due to expression of Cry protein, although there was no decrease in fungal, arbuscular-mycorrhizal fungal, or protozoan biomarkers. This suggests that Cry protein may have significantly reduced populations of soil microarthropods or other invertebrates. On the other hand, while the fungal biomarker is widely used, its validity is under question (Zelles, 1999). Research is currently underway to resolve these issues.

It is important to note that, while we have concluded that the impacts of Cry protein on the soil microbial community were limited in this experiment, further long-term experiments under a variety of conditions and using a variety of assays are required. Small impacts may be amplified in the field over time, and more detailed analyses may uncover other important changes in soil microbial communities. This point has also been made with reference to conventional pesticides, where effects on microbial community structure have been investigated relatively little using culture-independent methods (Johnsen et al., 2001; Kent and Triplett, 2002). The myriad of functions performed by soil microbes in agricultural systems makes this work essential to our ability to assess the sustainability of these practices.

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